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AN EXPANSION OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY
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THE EFFECTS OF THE MAJOR GENES CONTROLLING COAT COLOR IN THE GUINEA PIG ON THE DOPA OXIDASE ACTIVITY OF SKIN EXTRACTS[1]

BENSON GINSBURG
University of Chicago, Chicago, Illinois

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INTRODUCTION

THE approach to the problem of the genetic control of pigment formation has been a varied one, since the process of pigmentation provides at one and the same time a favorable and a difficult medium for the study of gene action. It is especially favorable in the mammal because it has been subjected to a more extensive genetic analysis than any other single character yet studied in this group. The guinea pig provides excellent material from this point of view, and WRIGHT (1916, 1917, 1925, 1927, 1941a, b, c, 1942) has made a more exhaustive study of the interaction effects of the major color genes in this form than exists for any other mammal. Moreover, the reaction from gene to end product is relatively short and is always occurring in certain specific cells which are readily accessible to the experimenter. In addition, extensive data have accumulated in the related fields of biochemistry, embryology, and endocrinology, but such studies have necessarily been restricted to one or a few genotypes and have consequently led to an oversimplification of the picture in some respects.

It has long appeared worthwhile to attack the problem of pigment formation by making a systematic study of the effects of all combinations of the known color genes on the physiological processes involved, thus combining the two possible approaches. A small contribution in this area could, conceivably, have large implications in the light of the data already made available by previous workers. This study involves such an attempt.

Genetic background

The major color genes of the guinea pig form seven series of alleles, none of them linked (WRIGHT 1941c). Different combinations of these alleles produce a gradation of visible pigmentation in the coat ranging from intense black or brown through various shades of sepia or light brown on the one hand, and from red through yellow and cream, on the other. WRIGHT (1927) has presented an analysis of these effects based on graded hair samples.

Later studies led to a quantitative refinement of the visible color grades by extracting the actual pigment and measuring it titrimetrically or colorimetrically. WRIGHT (1941b) has reanalyzed the effects of the color genes on the basis of the actual amount of pigment formed by the various genotypes as deter-

[1] This article is a revised version of a dissertation submitted to the faculty of the Division of Biological Sciences at the UNIVERSITY OF CHICAGO in candidacy for the degree of Doctor of Philosophy. The investigation was aided by a grant from the DR. WALLACE C. and CLARA A. ABBOTT MEMORIAL FUND of the UNIVERSITY.

mined by E. S. RUSSELL (1939) and HEIDENTHAL (1940). His tabular presentation of the quantitative relationships between pigment and gene perhaps is the best single summary of their effects.

Melanic animals (sepias or browns) differ from yellow and red (xanthic) individuals by possession of the dominant gene *E*. Some yellow is possible even in *E*— individuals if *A* is present. *E A* animals have a yellow subterminal band in each hair which behaves exactly as *ee* yellow with respect to modifier genes. Moreover, *E*—*ff pp* individuals have very pale pure yellow in the hair

TABLE 1

*The average concentrations of pigment in the hair of guinea pigs at birth as determined by Wright.**

	SEPIA		BROWN		YELLOW			EYE COLOR (*E*, *e*; *F*, *f* WITHOUT EFFECT)		
	EFPB *EfPB*	*EFpB*	*EFpb*	*EFPb* *EfPb*	*EfpB* *Efpb*	*eFPB* *eFPb* *eFpB* *eFpb*	*efPB* *efPb* *efpB* *efpb*	*PB*	*Pb*	*pB* *pb*
C—	100	21	17	50	6	100	36	Black	Brown	Pink
$c^k c^k$	90	18	15	42	0	38	5	Black	Brown	Pink
$c^k c^d$	82	15	13	47	0	41	5	Black	Brown	Pink
$c^d c^d$	64	9	11	40	0	38	5	Black	Brown	Pink
$c^k c^r$	94	14	13	43	0	19	0+	Black	Brown	Pink
$c^k c^a$	73	9	11	37	0	18	0+	Black	Brown	Pink
$c^d c^r$	75	7	7	44	0	14	0+	Black	Brown	Pink
$c^d c^a$	40	5	6	31	0	14	0+	Black	Brown	Pink
$c^r c^r$	84	5	6	45	0	0	0	Dk. Red	Dk. brown Red	Pink
$c^r c^a$	46	1	1	33	0	0	0	Lt. Red	Lt. brown Red	Pink
$c^a c^a$	0	0	0	0	0	0	0	Pink	Pink	Pink

* The sepias and browns are given on a scale in which intense black (*EPBC*) is 100. The yellows are given on a scale in which intense yellow (*eFC*) is 100. The latter has only about 20 percent as much capacity for reduction of $KMnO_4$ as intense black. Replacement of *a* by *A*, replaces the sepia, brown or yellow of *E*— combinations with the yellow of the corresponding *ee* combinations. The recessives are here represented by single symbols except in the *C*— series.

in the presence of *C*— and possess no color at all in combination with the lower alleles of the *C* series. The latter determines the intensity of whatever color is brought out by the other genes in a complex manner manifest as four levels of intensity in the yellow group, and as a series of waves in the sepias (see table 1) corresponding to the levels of yellow. WRIGHT (1917) has interpreted this as indicative of a difference in threshold for sepia and for yellow, and of competition between the xanthic and the melanic processes.

Replacements at the *P* and *B* loci are reflected by changes in the intensity of melanic pigmentation. Replacement of *F* by *ff* reduces yellow. *A*, *C*, *E*, *P*, and

B exhibit complete dominance, while *F* and the lower members of the *C* series are incompletely dominant over their alleles. e^p produces a tortoise shell pattern of red (or yellow) and melanic areas in the same animal. The gene *s* (not referred to above) determines the presence of white areas in spotted animals (these are also affected by nongenetic factors). Self colored animals are *SS*. The white-tipped condition sometimes found in the hair of intense brown has been ascribed to an eighth gene, *W*, by IBSEN (1932). This gene, or gene complex, produces a visible effect only in the genotype *C bb P* (WRIGHT 1942; EATON 1943). The relationship of these genes to eye color, though interesting, will not be discussed here, since it is not a part of this investigation.

Histology

Pigment is deposited as solid granules and is formed only in certain highly specific cells. These are the hair follicle cells and the dendritic cells found in the basal layer of the epidermis. The origin of these pigment-forming elements has not been clearly demonstrated for the guinea pig as yet, but it appears likely that they come from neural crest, since such an origin has been demonstrated for the pigment cells of several vertebrate groups by DU SHANE (1935, 1938), DORRIS (1938, 1939), EASTLICK (1939), and RAWLES (1940). The delimitation of the pigment-producing cells does not depend solely on observations of where the pigment granules are deposited, but may be accomplished by use of the dopa (3:4 dihydroxyphenylalanine) test as introduced by BLOCH (1917). Although BLOCH'S claims that a particular enzyme is at work in the pigment cells and that his dopa reaction is directly proportional to the pigment-forming activity have not been borne out by other workers, the fact remains that dopa may be used as a highly specific histochemical indicator of pigment cells in the mammal. Thus, it is possible to use the dopa test in order to isolate the cells involved in the pigment-forming process regardless of whether or not the theory behind its use is sound in all details.

A great deal of importance attaches to the specificity and determination of the melanophores and xanthophores. HAMILTON (1941) has offered evidence from tissue culture that argues for the view that the differences between cells capable of producing red pigment and those capable of producing melanic granules in the fowl are immutable after differentiation. WANG (1943) has shown that final differentiation occurs under the influence of the epidermis. Genetic evidence supports the concept of at least two qualitatively different systems (namely, black and red), though no more direct information exists for the mammal.

Physiology

It seems clear that the process of pigment formation involves the conversion of a colorless chromogen to a colored substance through a chain of oxidative reactions. This has led to the simple assumption that various degrees of pigmentation reflect corresponding changes in the stage of oxidation of the substrate. Thus "red" natural pigment has often been compared with the reddish phase through which dopa passes in its conversion into melanin. The oxidative processes could be controlled in various ways. It is possible that they depend

upon enzymatic activity, in which case one or several enzymes must be postulated and their sources determined. It is also possible that many phases of the reaction can occur in the absence of any such system. In such an event, oxidation-reduction potentials could be the determining influences (FIGGE 1940). As an alternative to these hypotheses, some evidence exists in favor of the presence of certain inhibitory substances which may merely be absent in the pigment cells, making it possible for oxidation of the substrate to occur and proceed to a stage determined by the quantity of inhibitor. The amount of substrate available may also be a limiting factor.

All attempts to analyze the pigment-forming processes have invoked one of these possible factors or some combination of them. BERTRAND (1896), BIEDERMANN (1898), and others were able to demonstrate tyrosinase activity in plants before the turn of the century. Inhibitory substances were also found. This led to the view that tyrosine was the substrate from which melanin is formed. RAPER (1928, 1932) later demonstrated that the more easily oxidizable dopa is an intermediate product in the artificial formation of melanin from tyrosine; since then several related diphenols and polyphenols have been used by workers as possible substrates.

The work of BLOCH (1927) led him and others to suggest the possibility of a specific dopa oxidase in the pigment cells. This provoked severe criticism based on the fact that dopa is too easily oxidized by nonspecific substances and that conditions other than the presence of a particular enzyme may have been involved. Part of this criticism arose from the early view that tyrosinases would oxidize dopa. Recent work by KUBOWITZ (1937) and by KEILIN and MANN (1938) has demonstrated that it is possible to prepare a highly purified polyphenol oxidase from extracts that originally showed marked tyrosinase activity. The purified fraction no longer exhibits such activity, nor is it a specific dopa oxidase. It is quite specific, however, toward a class of substrates—the polyphenols. BLOCH's "dopa oxidase" may be just such an enzyme.

FIGGE (1940) obtained evidence for the dependence of the dopa reaction on the oxidation-reduction potential of the system, there being inhibition by potentials beyond certain values in either direction from an optimum. He was able to produce similar results *in vivo* by keeping salamanders in dilute solutions of redox dyes. This led him to the conclusion that no enzyme is necessary for the conversion of dopa to melanin. Other evidence of a more circumstantial nature corroborates this view. CUNNINGHAM (1931) found that very small amounts of copper will exert a profound oxidizing effect upon dopa. The polyphenol oxidases recently prepared from different sources are high in copper content, and the amount of copper that may be extracted from mammalian skin and hair (SARATA 1935) is directly proportional to the pigmentation. It is entirely possible, therefore, that copper acts as a catalyst to the reaction, or, more probably, that the enzyme itself is a copper proteinate. The essential role of copper, however it may act, is illustrated by recent work on nutritional achromotrichia (KEIL and NELSON 1931) where hooded rats kept on a copper-free diet lost pigment that was promptly restored when the deficiency in the diet was corrected.

A great deal of additional information exists with respect to other dietary factors, ultraviolet light, hormones, and activators. These have not yet been sufficiently related to the genetic mechanism to warrant discussion here.

Genetic control of the physiological processes

Two methods have been used with some success in relating the genes to the physiological mechanism of pigment production. Both depend upon the supposition that an enzyme system is basic to the melanogenic processes and that some of the color genes act by controlling the level of enzyme production.

The first of these involved attempts to prepare extracts from genetically known materials and to test them for possible enzymatic activity toward supposed pigment precursors. Earliest attempts were made using tyrosine as a chromogen. Later, more successful attempts were made using dopa, though no conclusive data were obtained with either substrate.

DURHAM prepared aqueous extracts of the skins of young black rats, rabbits, and guinea pigs as early as 1904. She used tyrosine as a chromogen and ferrous sulfate (supposedly as an activator, though ONSLOW (1915) later demonstrated that the reverse was actually true—that is, ferrous sulfate inhibits the reaction). She reported differences between color types, but ONSLOW was unable to confirm her results. In following the then extant theory of BACH and CHODAT (who held that oxidases consist of a peroxide and a peroxidase) ONSLOW prepared chloroform extracts of rabbit skins and tested these against tyrosine at a slightly alkaline pH in the presence of hydrogen peroxide. ONSLOW also claimed positive results for dark-skin extracts, but PUGH (1933) was unable to confirm him. CHARLES (1938) observed two cases of possible tyrosinase activity in extracts made from the black areas of skins of spotted mice, but only after long incubation.

KOLLER (1930) used dopa as a chromogen and obtained results similar to ONSLOW's. He did not state his experimental pH, nor were his controls adequate.

Both KAUFMANN (1925) and KOSSWIG (1927) were unable to repeat ONSLOW's results. The latter worked with skins of black rats and with lymph from the skins of black rabbits. He was unable to obtain positive results with either tyrosine or dopa. As a matter of fact, his extracts inhibited the auto-oxidation of dopa.

DANNEEL and SCHAUMANN (1938), faced with these mutually contradictory results, attempted to repeat some of them and to carry the work to a point that would clarify this phase of the problem. Their repetition of ONSLOW's work gave wholly negative results. When KOLLER's method of replacing tyrosine by dopa was used, and adequate controls were run, it was found that not only were the extracts inactive, but they inhibited the auto-oxidation of dopa. On the basis of their own work they concluded that the extracts consisted of an inhibitor, produced by the extraction and contained in the albumin fraction, and a dopa oxidase, contained in the globulin fraction.

The second method used to relate the physiological and the genetic mechanisms involves treating frozen sections of skin with properly buffered chromogen

and observing the histochemical results. SCHULTZ (1925) used this method with rabbits, and KRÖNING (1930) found it applicable to guinea pigs. W. L. RUSSELL (1939) made an extensive investigation of genotypes of the guinea pig, using animals bred by PROFESSOR WRIGHT. APPEL (unpublished) has confirmed his results.

In general, RUSSELL found that the reactions in the basal layer of the epidermis were different from those exhibited by the hair bulb. The *C* series affected the dopa reaction in both areas. Four levels of intensity were distinguishable in the hair bulb following the order shown by the effects of this series on yellow in table 1. The strongest reaction was obtained with *C*, a lower one with any of the group c^kc^k, c^kc^d, or c^dc^d, still lower with c^kc^r, c^kc^a, c^dc^r, or c^dc^a, and none at all with c^rc^r, c^rc^a, or c^ac^a. On the other hand, *C* was necessary for any reaction at all in the basal layer. No reaction occurred in white regions of *ss* (spotted) animals that gave a strong reaction in the black areas. Replacement of *E* by *ee* reduced the hair-bulb reaction slightly but had no effect on the basal layer; *ff* had a stronger but parallel action. On the other hand, *pp* markedly reduced the basal layer reaction but did not seem to make any difference in the hair bulb. This could indicate that two enzyme systems are present, both capable of catalyzing the dopa reaction, but each concerned with only the black or the yellow phase of pigment production. The hair-follicle cells gave only reactions which paralleled yellow pigment production regardless of the color of the animal. The reverse was true in the basal layer.

Other interpretations are also possible as indicated in the previous section. The observed effects of the genes on the dopa reaction may be due to effects on non-enzymatic conditions that regulate the process, and there may be no enzyme system whatever involved during the dopa-to-melanin phase of pigment formation. The frozen-section method cannot clarify the problem beyond providing a basis for the demonstration of gene-controlled differences and showing where these occur. If an enzyme system is involved, its analysis depends upon the evolution of suitable methods of extraction and purification.

MATERIALS AND METHODS

The test solutions

Of the two pigment precursors most generally used, only dopa has given favorable results that can be relied upon. It was, therefore, used in these experiments in the concentration found adequate by RUSSELL (1 mgm/cc buffer solution). 2M/15 phosphate buffers were used and the pH adjusted to 7.4 and tested before each experiment. Phosphates afford adequate protection against changes in pH and do not interfere with the dopa reaction as borax buffers do. Carbonates, though usable, are less stable on contact with air. Ordinary distilled water (tested for electrical resistance) was used in most of these experiments. A few were run with double distilled water, but this method was soon discarded as no differences were noted in the results.

The pH was fixed at 7.4 and the incubation temperature at 40°C in order to approximate the conditions under which the reaction must take place in the

guinea pig. Both conditions are favorable for the dopa reaction, but neither is optimal.

The final ratio between extract and dopa solution was fixed at 1:1 after several other relationships had been tried because this gave maximum differentiation between white and black animals with any given extract. One-half cc of each was used in order to conserve materials. This amount is adequate.

An incubation period of six hours was used in the early experiments, but this proved too long because some settling out of dopa melanin could easily be forced by short centrifugation after such an incubation period. This was undesirable from the point of view of colorimetric assay, so after a number of trials, a two hour period was adopted.

Dopa itself is easily oxidized when exposed to air in an alkaline medium and at body temperature so that it changes from a colorless compound through pink and purplish-brown intermediate stages to a dark colored solution in four to six hours at the concentration used in these experiments. The almost black dopa melanin will then aggregate and precipitate out on further standing. In order to judge the effect of the extract as compared with the auto-oxidation of dopa, a control consisting of one-half cc buffered dopa+one-half cc distilled water was always included in each experiment.

Genotypes

All animals used were taken from matings made by PROFESSOR SEWALL WRIGHT, and genotypes were determined by him from the pedigrees. Results obtained with animals of questionable genotype were not included in the final tabulations.

Technique of extraction

Animals 30 days old or older were killed with ether, clipped, and skinned by making a mid-dorsal incision. All subcutaneous tissue was removed by scraping with a knife, and the skins were washed under cold, running tap water for one to two hours. They were then superficially dried, reduced to a standard weight by discarding any excess, individually wrapped in cheesecloth, and frozen solid in a refrigerator overnight. After this they were ground in a meat grinder and mixed with one-half their weight in chloroform and one-tenth their weight in infusorial earth. This pulp was thoroughly mashed in a mortar, wrapped in cheesecloth, and any contained fluid was expressed by means of an efficient cast-iron screw press. This was filtered twice through double layers of filter paper and a plug of infusorial earth to give a final extract that was slightly opalescent to the eye. The extracts were allowed to stand overnight in a refrigerator (chloroform added as a preservative) and were used the following day. These were termed the "immediate" extracts. In several instances extracts were not used until one or four days after the usual interval of standing. These were termed the "delayed" extracts.

Buffered dopa was freshly prepared before each experiment, and reaction mixtures of dopa and extract were made up as indicated above. These were incubated in cotton-plugged test tubes for a period of two hours at 40°C. They were then read by visual comparison and in the Evelyn photoelectric colorimeter (EVELYN 1936).

All such tests made with white animals, regardless of the genetic reason for their whiteness, inhibited the auto-oxidation of dopa for eight hours or more. There was frequently a just discernible increase in opalescence, but rarely any trace of color visible to the eye. As brought out later, some color could be detected by colorimetry. In contrast, the auto-oxidized control was always quite dark. Extracts prepared from *C−FF* animals were often considerably darker than the control (by visual inspection) but were as frequently at control level or even lighter. All intermediate reactions between this and complete inhibition were obtained, depending on the genotype being tested.

Measurement of the reaction

The relative concentrations of dopa melanin formed as a result of incubating the dopa solutions with extracts prepared from various sources were determined by photoelectric colorimetry. Visual colorimetry, employing a Klett biometer, would have been impractical because it is exceedingly difficult to prepare suitable standards and because the subjective error is relatively great with very light-colored materials. Duboscq colorimetry, while accurate, also has the disadvantage of requiring standards. The Evelyn photoelectric colorimeter has none of these disadvantages and affords a rapid method for the determination of the concentration of any colored substance in solution for which a suitable filter can be found. Such a filter must be sufficiently selective to make the laws of Lambert and Beer applicable. Spiegel-Adolf (1937) showed that these laws could be applied to melanin solutions.

The method involves the passing of monochromatic light (in this case between 3800 and 4600 Å) through control and test solutions. The intensity of the light source was adjusted so as to give the maximum galvanometer reading on passage of the light through a distilled water control. This was followed by a galvanometer reading of the test solution (made up with dopa and extract). Readings of the colorless solutions containing extract from white skin showed some absorption of light due to murkiness. Allowance should obviously be made for this in determining the amount of dopa melanin produced in the tests of colored skin. It was at first assumed that the tests of extracts from whites could be used for this purpose. This turned out to be rather unsatisfactory because of variability and because the light absorbed by such solutions increased with time. In the later tests a galvanometer reading was obtained for each extract from a control containing the extract at the standard dilution, but without dopa.

The transparency (T) of a test solution is measured by the ratio of the intensity of the transmitted light (I) to that of the incident light (I_0): $T = I/I_0$.

The amount of light that escapes absorption falls off exponentially with the concentration (C) of the absorbing substance: $T = e^{-KC}$, $C = (1/K) \log_e (1/T)$, where K is a constant.

The optical density (L), as conventionally expressed in photoelectric colorimetry is defined as follows: $L = \log_{10} (1/T)$. It is obviously proportional to the concentration.

The galvanometer reading is proportional to the intensity of the light striking the photocell. As the galvanometer is adjusted to give the maximum

reading of 100 (with each filter) in the case of the blank, the reading for a given solution (and filter) needs merely to be divided by 100 to give the value of T after a correction has been made to eliminate the non-linear relationship between the amount of light incident upon the photocell and the current actuated by that cell: $T = G/100$ and $L = \log_{10}(100/G) = 2 - \log_{10} G$.

All readings are given in terms of the Activity Index, A, derived as follows: L_R = optical density of the chromogen in the test solution (dopa+extract).

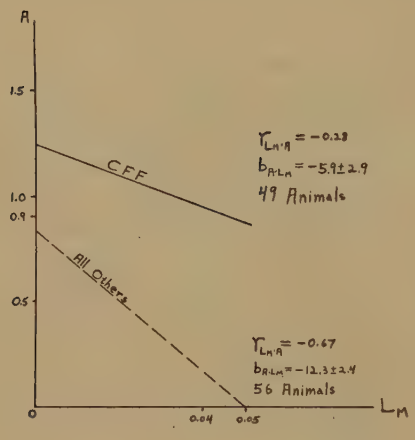

FIGURE 1.—A regression line comparison between the dopa oxidase activity (A) and the concentration of extracts as measured by their murkiness (L_M).

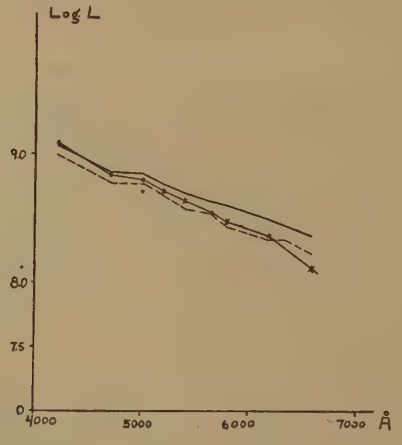

FIGURE 2.—Absorption spectra of dopa melanins formed by the action of three different oxidizing agents on dopa. ———— Auto oxidation (two samples). —×—×— Potato extract (three samples, each prepared from a different potato). ---------- Guinea pig extract (nine samples, each prepared from a different animal). All melanin samples in this and the following figures were dissolved in 0.2 N KOH unless otherwise indicated.

L_M = optical density due to the murkiness of the individual solutions (control containing extract but not dopa). In the earlier tests, in which there were no individual murkiness controls, the average of all of later murkiness readings was used as L_M.

L_D = optical density of dopa melanin in the control (containing dopa but not extract). $A = (L_R - L_M)/L_D$.

This index shows at a glance whether the extract has been largely inhibitory (A less than 1) or enzymatic (A greater than 1) in effect. It must be remembered in this connection that extracts reading less than one may show various degrees of activity above that shown by extracts from white even though below the level of auto-oxidation. We shall anticipate the conclusion of later discussion by referring to such activity as enzymatic.

There seems to be a measurable relationship between the murkiness of the extracts as defined above and their activity (fig. 1). Correlation coefficients

between the activity index (A) and the murkiness index L_M were obtained for 49 animals of genotype $C-FF$ and for 56 animals of other genotypes shown later to be less active. In all of the former, L_M was 0.05 or less. There were six cases in the second category in which L_M was higher and activity was virtually absent. These are excluded, because if 0.05 is the point of practically complete inhibition, and if all values beyond this point show virtually no activity at all, there is obviously no justification for flattening the slope of the regression line.

In the extracts prepared from animals producing little or no enzyme the correlation between A and L_M was -0.67, and the regression of A on L_M was -12.3 ± 2.4. If the extracts are almost water clear, the activity (about 0.8) tends to approach the level expected from auto-oxidation (1.0). As murkiness increases, the activity declines sharply in an approximately linear fashion to the point $L_M = 0.05$ at which A practically reaches zero.

Extracts obtained from animals of genotype $C-FF$ also showed greatest activity (about 1.25) when the murkiness was negligible, but this level of activity was relatively slightly reduced as the murkiness increased ($r = -0.28$, $b = -5.9 \pm 2.9$), suggesting that both enzyme and inhibitor are concentrated in the less clear solutions, but that the inhibitory activity slightly outweighs the enzymatic effect. When no enzyme is present, only inhibitor is concentrated as murkiness increases, and the slope of the line representing this relationship is consequently much steeper. This seems to be the most plausible of the possible explanations in the light of the evidence (to be discussed later) for the existence of a gene controlled enzyme and of a separate but naturally occurring inhibitor whose concentration is apparently not a function of the color genes.

The relatively wide variation in the opalescence of the extracts is not related to either the age or the genotype of the animals. It accounts for a substantial portion of the variability of the final readings. Since the regression lines (fig. 1) are based on large composites of various genotypes (table 2), no one of which could be separated out with confidence because of small numbers, and each of which may have its own characteristic slope, it becomes impracticable to apply a correction factor based on the regression coefficients in order to equate the concentrations of enzyme and inhibitor in all extracts to a common denominator.

All series are otherwise strictly comparable. Each 1-cc reaction mixture (and control) was diluted to 10 cc with the buffer mixture and was read in the colorimeter at this dilution. Corrections were made as described.

Absorption spectra of the melanins[2]

In order to know how the genes affect the color-producing system, it is desirable to know whether the end products differ qualitatively or quantitatively

[2] Baker and Andrews (1944), in a paper which appeared while the present article was in press, report differences between light absorption curves of red and black guinea pig melanins which are in agreement with those obtained from this study. Their interpretations differ from those given here in being predicated on the assumption that red pigment is more highly oxidized than black, and that the *E* gene must therefore determine a lower level of oxidation than the gene combination *ee*. Direct measurements of the oxidative capacities of the pigment-forming cells in these genotypes as made by W. L. Russell (1939) show that the reverse is actually true.

from each other. Different interpretations are necessary for either alternative.

Many studies made with the melanins indicate that there is no difference between them that can definitely be ascribed to a different molecular structure. These studies are deficient in two respects: (1) No really good methods of obtaining clear solutions of melanin were developed until recently. (2) No really pure intense red pigment was used in any analysis known to this writer that was undoubtedly free from contamination by sepia pigment.

The problem, therefore, is still open. Thanks to the efforts of E. S. Russell (1939), Heidenthal (1940), and Ivaska (unpublished), improved methods were developed for extracting the different pigments. As is well known, the

Table 2

Distribution of some of the major genotypes with respect to murkiness (L_M) and dopa oxidase activity (A).

GENOTYPE	NUMBER OF ANIMALS	L_M	A
ee aa C−FF P−B− aa	7	0.021±0.005	0.69±0.10
E−A−C−FF P−B−	20	0.021±0.003	0.92±0.08
E−aa C−FF P−bb	17	0.026±0.005	1.22±0.10
E−aa C−FF pp B− e^p	5	0.039±0.010	0.98±0.03
E−aa C−Ff P−B− ee	10	0.018±0.005	0.70±0.14
E−aa C−ff P−B−	12	0.022±0.005	0.56±0.11
Combinations with lower alleles of the *C* series.	39	0.028±0.003	0.32±0.04

blacks and reds differ greatly in ease of extraction, a fact which may depend either on a chemical difference or on the density of the granules. Once in solution, the color is surprisingly similar. The reader is referred to the above papers for a fuller discussion of pigment solutions.

Daniel (1938), using a König-Martens spectrophotometer, was unable to find any reliable difference in the absorption spectra of the various melanin pigments. However, she had no pure red samples to work with. Accordingly, spectra were determined for solutions of pigment extracted from guinea pig hair and read in the Evelyn colorimeter. Each sample was read twice at each of ten points on the visible spectrum (fig. 2–6) by the use of suitable filters. The readings were then converted to $\log_{10}$ optical densities ($\log_{10}$ L) and plotted as such in order to achieve a direct comparison between percentage differences.

Ten cc aliquots of each solution to be tested were placed in the colorimeter tubes for analysis as described above. In some cases, water was used as a solvent. Resulting curves showed very little slope and a suspensoid sometimes formed which was not very satisfactory for reading because of the large size of the suspended particles. Two-tenths N KOH was used as a solvent in all other cases.

Dopa melanin in any stage of oxidation or however derived, tyrosine melanin at any stage of oxidation, potato-melanin and dissolved pigment from black or sepia pigs (all samples of guinea pig pigment were prepared by Miss Ivaska) have similar spectra. This is compatible with the notion that dopa

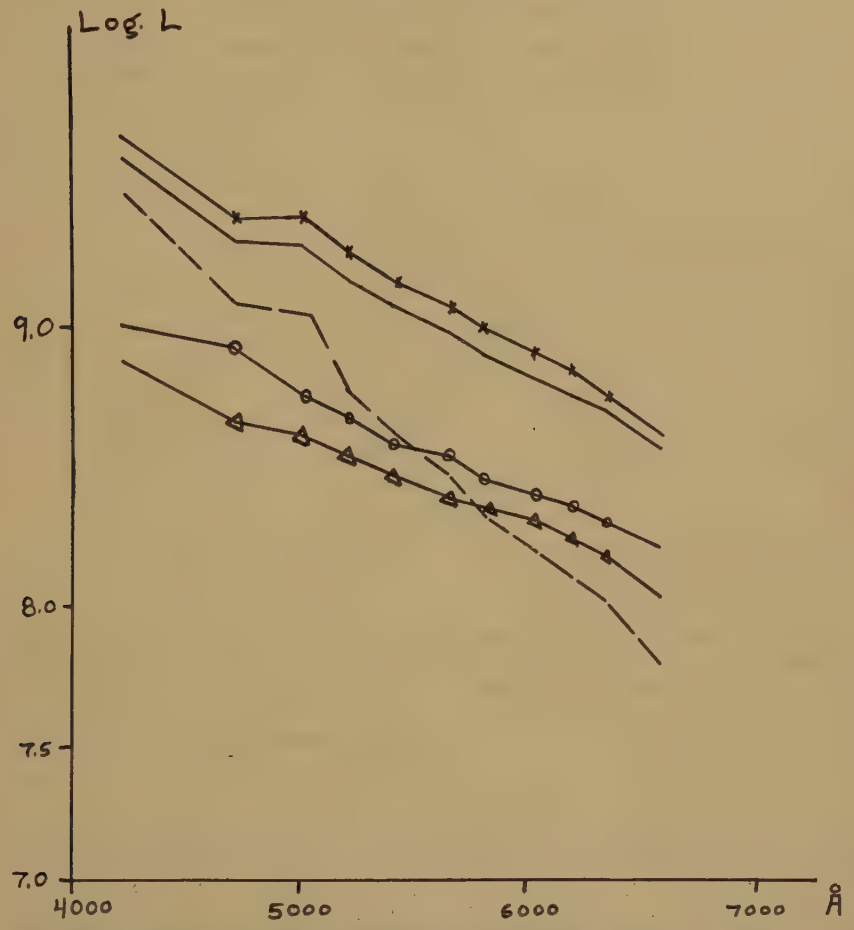

Figure 3.—Absorption spectra of various natural and artificial melanins. —×—×— Natural melanin extracted from the hair of eight different black guinea pigs (*E—aaC—FFP—B—*).* ———— Natural melanin extracted from potato peelings. ---------- Natural melanin extracted from the hair of three different red guinea pigs (*eeaaC—FF—B—*).* —○—○— Artificial dopa melanin (14 samples).* —△—△— Artificial tyrosine melanin (two samples).*

* Each point on the spectra in the accompanying figures is based on the average of independent readings made as described in the text for each sample separately.

melanin, tyrosine melanin, and natural melanin pigment, wherever found, are one and the same substance, and that lighter and darker shades of melanic pigmentation are essentially nothing but quantitative variations. It is also entirely possible that any differences that might exist between the solutions of

artificial pigment at early and at later stages of oxidation were masked by the occurrence of a small amount of completely oxidized (black) pigment at a time when the solutions still appeared to be light pink or red. Natural red pigment from genetically red pigs, however, gives a curve with a much steeper slope. The occurrence of demonstrable impurities in the solutions renders a more detailed and rigorous comparison unreliable for the present.

It is impossible to state the nature of the difference between natural red pigment as it occurs in the guinea pig, and all the other samples tested at the

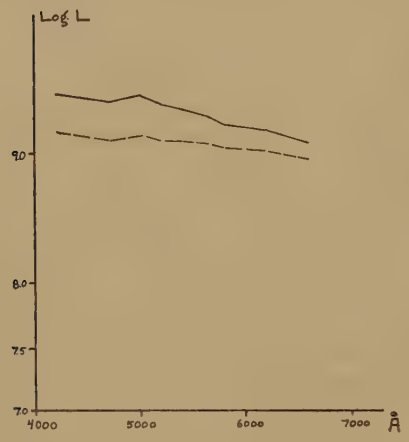

FIGURE 4.—Absorption spectra of dopa and tyrosine melanin in aqueous solution. ———— Tyrosine melanin (three samples). ---------- dopa melanin (thirteen samples).

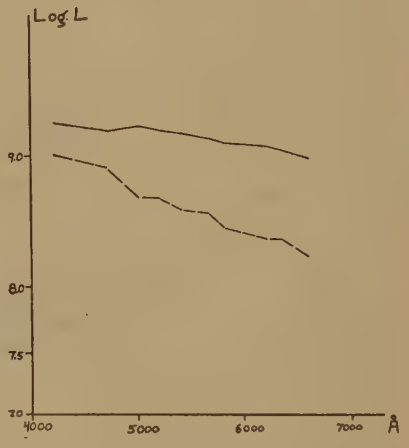

FIGURE 5.—Absorption spectra of dopa and tyrosine melanin in different solvents. ———— Dopa and tyrosine melanin in aqueous solution (sixteen samples). ---------- Dopa and tyrosine melanin dissolved in 0.2 N KOH (sixteen samples).

present time. Both curves show maximum absorption at the violet end of the visible spectrum and a rapid decrement with increasing wave length that is definitely greater in the case of pigment from red guinea pigs than in that from the other sources. There is no well-defined absorption band in the visible spectrum in either case, although both show a slight hump in the blue-green area, the significance of which requires further study. Whether the difference described above is to be attributed to chemical structure or to physical state in these colloidal solutions is uncertain.

MAJOR RESULTS

Initial considerations

Preliminary indications (based on table 3) are that all whites, regardless of genotype, yield an extract that almost completely inhibits the dopa reaction under the conditions mentioned. This includes the white areas of spotted animals (*ss*), the white of albinos ($c^a c^a$), and the white of red-eyed whites (*ee* $c^r c^a$).

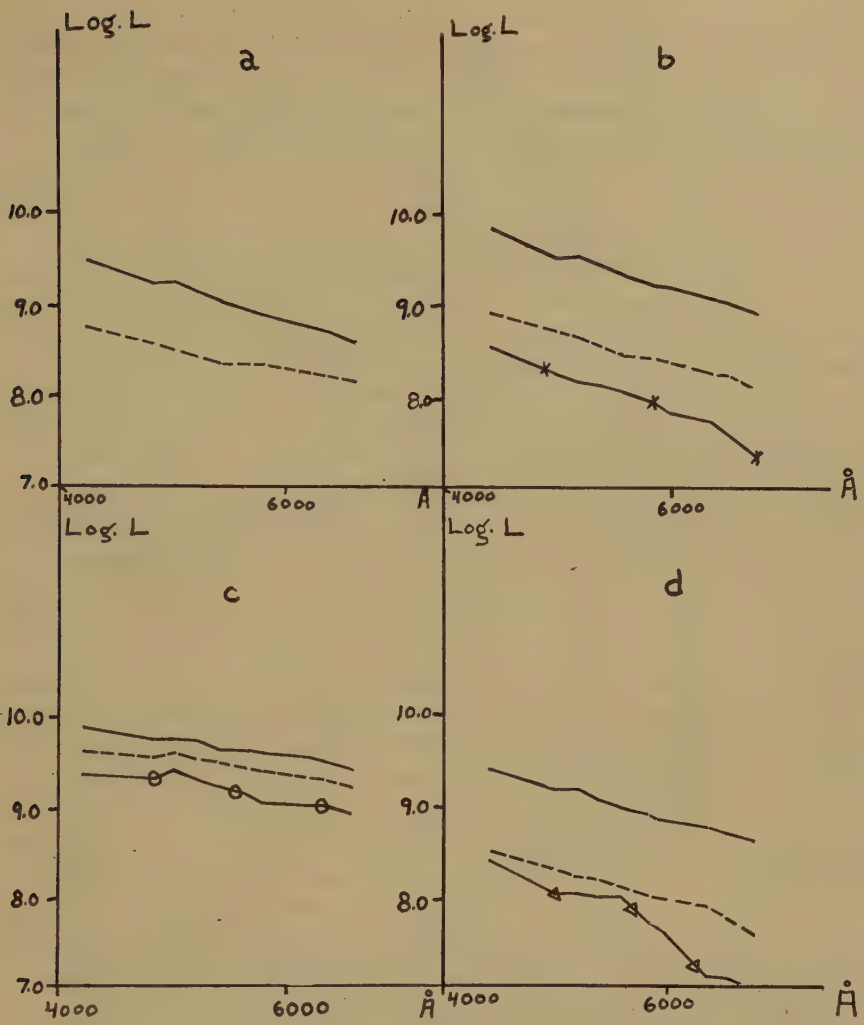

FIGURE 6.—Absorption spectra of dopa and tyrosine melanin at various stages of oxidation.

FIGURE 6a.—Auto oxidized dopa. ———— After 29.5 hours at 40°C.
---------- After two hours at 40°C.

FIGURE 6b.—Dopa melanin formed by the oxidizing action of potato extract. ———— After four hours at 40°C. ---------- After one hour at 40°C. —×—×— Immediately after addition of colorless, filtered extract.

FIGURE 6c.—Tyrosine melanin (in aqueous solution) formed by the oxidizing action of potato extract. ———— After four hours at 40°C. ---------- After one hour at 40°C. —○—○— Immediately after addition of extract.

FIGURE 6d.—Tyrosine melanin (in 0.2 N KOH) formed by the oxidizing action of potato extract. ———— After four hours at 40°C. ---------- After one hour at 40°C. —△—△— Immediately after addition of extract.

Note: All extracts were pigment free as judged by colorimetric tests.

At the other extreme are the strong reactions given by the extracts from most of the blacks, browns, and some of the paler types. Some blacks and browns, however, give extracts that almost completely inhibit the dopa reaction. In fact, it is impossible to correlate the level of black, brown, or sepia pigmentation with the dopa reaction given by extracts from such skins. Some

TABLE 3

Genetic levels of enzyme production.
(All readings are in terms of A values *cf* page 184)

GENOTYPE	COLOR	IMMEDIATE				DELAYED			
		NO.	MEAN	STANDARD DEVIATION	S.E.	NO.	MEAN	STANDARD DEVIATION	S.E.
$E-^{1}aaC-FFP-B-$	Black	25	0.97	0.34	0.06	5	1.68	0.61	0.30
$E-A-C-FFP-B-$	Agouti	7	0.92	0.38	0.11	3	1.18	0.23	0.31
$E-^{1}aaC-FFP-bb$	Brown	14	1.09	0.24	0.09	13	1.98	0.82	0.21
$E-aaC-FFppB-$	Pale sepia	7	0.92	0.18	0.11	3	1.74	0.94	0.40
$E-^{1}C-FF$	—	53	0.99	0.35	0.05	24	1.79	0.80	0.16
$E-aaC-Ff$	Melanic[2]	8	0.78	0.42	0.10	1	1.18	—	—
$E-aaC-ff$	Melanic[2]	4	0.51	0.21	0.12	3	1.00	0.38	0.28
$ee^{3}C-FF$	Red[4]	9	0.65	0.27	0.08	5	0.94	0.07	0.21
$eeaaC-Ff$	Red[4]	7	0.53	0.25	0.09	—	—	—	—
$ee^{3}aaC-ff$	Yellow[4]	9	0.27	0.20	0.06	1	0.75	—	—
$e^{p}c^{k}c^{k}FF$	Dilute[5]	5	0.29	0.25	0.09	2	0.56	—	—
$-c^{kd}c^{ra}FF$	Dilute[6]	27	0.30	0.18	0.04	12	0.37	0.62	0.09
$-c^{r}c^{ra}FF^{7}$	White or dilute brown[6]	7	0.28	0.26	0.07	3	0.37	0.13	0.18
$-c^{a}c^{a}FF$	White (from albinos)	12	0.16	0.16	0.05	6	0.34	0.23	0.12
$ss-$	White (from spotted animals)[8]	5	0.30	0.10	0.09	3	0.20	0.05	0.15
Below *C* or white of *ss*	—	56	0.27	0.21	0.03	26	0.37	0.19	0.03
		146 Animals				60 Animals			

[1] Includes some $e^{p}e^{p}$ in which black or brown areas from tortoise shells ($e^{p}e^{p}$) were used.
[2] Includes black ($P-B-$), pale sepia ($ppB-$), intense brown ($P-bb$), and pale brown ($ppbb$).
[3] Includes some $e^{p}e^{p}$ where red or yellow areas from tortoise shells ($e^{p}e^{p}$) were used.
[4] Includes both black-eyed ($P-B-$) and pink-eyed ($ppB-$) animals.
[5] Includes yellow-agouti ($A-P-B-$), yellow ($aaP-B-$), black ($aaP-B-$), brown ($aaP-bb$), and pale sepia ($aappB-$).
[6] Includes blacks ($aaP-B-$), sepias ($aaP-B-$), and creams (ee).
[7] Includes whites ($eeB-$) and dilute brown ($E-bb$).
[8] Four of these were $E-C-FF$.

very lightly pigmented animals are capable of producing a reaction fully as strong as that obtained from much darker specimens, while some of the latter produce strong inhibition as cited. These differences, which seem impossible to interpret from the standpoint of quantity of pigment, fall into order when considered from the standpoint of genotype. Further order is obtainable by distinguishing the cases in which the extracts were used immediately from those in which they were held over for one or more days, since it appears that such delay in the use of the extracts produces a variable enhancement of the oxidative effects of the solutions (see table 3).

Effects of the genes on the dopa reaction

The relative effects of the extracts, based on genotype, are brought out by the groupings in table 3. Since the numbers in many of these groups are small, the standard deviations (table 3) cannot be relied upon as a basis for calculating meaningful standard errors. Neither can the average intra-group standard deviation be used, because the magnitude of each standard deviation tends to be proportional to the size of the mean. Smoothed estimates of the standard deviations were accordingly obtained from the regression of standard deviation on mean ($r=0.64$, $b=0.16\pm0.02$ in the case of the "immediate" tests; $r=0.65$, $b=0.28\pm0.04$ in the "delayed" tests).

It appears that there are no important differences among the four groups showing greatest activity or among any of the subgroups within these. Comparisons not shown here indicate that there is no difference in dopa reaction between extracts from black or brown areas from tortoise shells (e^pe^p) and those from black or brown self colored animals ($E-$). As illustrated in the table, replacements at the *P*, *B*, or *A* loci do not affect the reaction significantly though *pp* pale sepias have only 21 percent as much pigment as black animals, and the intense browns (*bb*) have only 50 percent even when white-tipping does not occur. Most of the browns tested were very much lighter, in fact, because of white-tipping. All of the above produce a strong dopa reaction as long as $E-C-FF$ are present, the average being 0.99 ± 0.05 for the cases in which the extracts were used immediately. The reactions in "delayed" tests are irregular but significantly higher. It is noteworthy that the average amount of melanin produced in the latter is above that produced on auto-oxidation of dopa during the same interval, and that some individual readings give A values of over 4.00.

Red areas of both $eeC-FF$ and e^pe^pC-FF animals give a significantly lower reaction than is obtained for any of the groups cited above (average $=0.65\pm0.08$).

The replacement of *FF* by *ff* reduces the dopa reaction drastically (from an average value of 0.99 ± 0.05 to one of 0.51 ± 0.12 for the sepias and browns, in spite of the fact that there is no visible difference between the coat colors of *FF* and *ff* melanics; and from 0.65 ± 0.08 to 0.27 ± 0.06 in the case of the already somewhat reduced reds, where *ff* is visibly much lighter than *FF*). *Ff* is intermediate in both cases, giving an average of 0.78 ± 0.10 in the sepias and browns, and one of 0.53 ± 0.09 for the reds. It is to be noted that $E-$ (or e^p in the melanic areas of tortoise shells) shows more activity than *ee* (or e^p in yellow areas of tortoise shells) not only in the presence of *FF*, but also in combination with *Ff* or *ff*. In yellows of genotype *eeff* there appears to be no activity at all (0.27 ± 0.06 as compared with 0.27 ± 0.03 in the case of whites and others exhibiting negligible activity). Therefore, we must postulate a systematically lower level of oxidative activity for reds and yellows (*ee* combinations) than we do for blacks, browns, and sepias ($E-$ combinations) of otherwise similar genotypes.

In the presence of *FF* it is possible to produce a reduction in the dopa reaction if lower members of the albino series are substituted for $C-$. It has

already been noted that there the minimum reaction, approaching complete inhibition, is found in albinos (c^ac^a) and eec^rc^a whites. This was also the case in Eec^rc^a browns, although these have considerable more pigment than pale sepias of genotype $E-C-FFpp$ which are highly active. It is doubtful, moreover, whether there is any reaction at all even when c^kc^k is the highest combination of the series present if the tests are made immediately. No effect is obviously demonstrable from the average (0.29 ± 0.09) derived from the blacks, browns, yellow-agoutis, sepias, and yellows of this group. If, however, the delayed extracts are considered, it becomes apparent that it may be possible to concentrate the active substance in such combinations on standing. No similar concentration is possible with the *ss*, c^ac^a, or eec^rc^a whites. Delayed $c^{kd}c^{ra}$ blacks, sepias, and creams give occasional readings as high as 0.60, though their average is only 0.37. There is, therefore, the possibility of the production of an active agent in all colored areas of *FF* animals carrying c^k or c^d as their highest albino allele. No such activity was exhibited by extracts made from the skins of any of the white animals or from white regions of spotted animals, even on standing. When only the "immediate" group is considered, there is no evidence for treating any combination of alleles below *C* in the albino series as a separate category. The entire group may be considered an entity for purposes of comparison with other genotypes. Standard errors for this and the large melanic composite (including black, agouti, brown, and sepia) were calculated from the entire data in the manner already discussed by regrouping and by fitting new regression lines ($r=0.93$, $b=0.19 \pm 0.01$ for the "immediate" and $r=0.95$, $b=0.44 \pm 0.02$ for the "delayed" extracts).

If the reader prefers to focus on any subgroup as the baseline or theoretical zero, it would be best to consider the *ss* animals in this light, since there seems to be no possibility of pigment production in the white areas of the coats of such animals under any circumstances, while some pigment is normally produced in all the other genotypes, even including the c^ac^a whites (albinos) on exposure to cold.

Comparison with the dopa reaction in frozen section

The results described above for extracts are closely parallel to the dopa reactions of the pigment cells of the hair follicles as described by W. L. Russell —namely, strongest reaction with $E-C-F-$, a slightly reduced reaction with $eeC-F-$, a greatly reduced reaction on replacement of *F* by *ff* or of *C* by c^k or c^d, and no reaction in the white areas of spotted animals (*ss*) or on replacement of *C* by c^r or c^a. The results also agree in the absence of appreciable effects of replacement of *aa* by *A*, of *P* by *pp*, or of *B* by *bb*. The imperfect dominance of *F*, indicated by the present data, was not studied by Russell.

On the other hand, there is no parallelism with the dopa reaction of pigment cells in the basal layer as described by Russell. This investigator found an appreciable reaction in the basal layer only in colored areas of animals with $C-P-$. Replacement of *F* by *ff* as well as of *E* by *ee*, *aa* by *A*, and *B* by *bb* was without apparent effect. Replacement of *C* by any lower allele or of *P* by *pp*

PLATE 1 (above).—Section through the skin of a white guinea pig (×40) lightly stained to show the epidermis.

PLATE 2 (below).—Section through the skin of a white guinea pig (×66) from which the epidermis has been removed by scraping.

virtually eliminated the reaction. RUSSELL concluded that there are two active principles, one demonstrable (under the conditions of the experiment) in follicles, the other in the basal layer.

It may be concluded that in extracts prepared under the conditions described above the positive activity is completely dominated by substances from the follicles rather than from the basal layer.

Comparison with the phenotypes

The dopa reactions of the extracts as well as of the follicle cells *in situ* agree closely with the effects of the genes on yellow pigment. Browns, sepias, and blacks give reactions agreeing with the intensity of the corresponding yellows (obtainable by replacement of *E*— by *ee*) and not at all, as emphasized repeatedly, with the actual quantities of melanin pigment in these animals. It should be pointed out that there is one result which is not explained by this hypothesis—namely, the higher activity with *E*— than with *ee*. These should give the same reaction, under the hypothesis that the active agent is the yellow producing system, present alike with *E*— and *ee* unless, perhaps, more is extractable with *E*, where it is not actively engaged in producing yellow pigment, than with *ee*, where it is. More work is clearly necessary before any such explanation is accepted.

The reaction in the basal layer observed by RUSSELL parallels to some extent the intensity of black, but, as noted, there is no evidence that this active principle was present in the extract. It is possible that it is thermolabile, even at the temperatures reached in these experiments, or that it is not, in reality, a chemical substance.

A seeming paradox presents itself here. The intensity of the dopa reaction *in vitro* (under the conditions already described) seems to be determined entirely by a substance concerned with yellow pigmentation, yet the absorption spectra of the resulting dopa melanin are indistinguishable from that of natural black pigment and are definitely different from that of natural yellow pigment in the preparations tested. It is possible, however, that dopa is not the natural chromogen, at least for yellow, and that the dopa reaction is merely an indicator of the concentration of a somewhat nonspecific agent. An alternative possibility which is, perhaps, preferable is that the difference in the spectra of black and yellow pigment is purely physical, reflecting a difference in the mode of deposition and aggregation of the pigment in granules and in the colloidal particles derived from these in the solutions. It may happen that the reaction *in vitro* produces melanin in a physical state more closely resembling that from solutions of black granules than from yellow (red) granules.

EXPERIMENTAL ANALYSIS OF THE EXTRACT

Attempts to remove the inhibitor

DANNEEL and SCHAUMANN (1938) state that they obtained positive results with the extract method only after removing the inhibitory substance in rabbit extracts by aeration, the addition of peroxide, or fractional salting out.

The first two methods did not give systematic results with the guinea pig, though many attempts were made. Fractional salting out produced a globulin fraction showing high dopa-oxidase activity, but in no case did the albumin fraction contain the inhibitor as DANNEEL and SCHAUMANN state for the rabbit. After dialysis (to remove the ammonium sulfate used in salting out) albumin was completely inactive in either direction. (It was largely so before dialysis as well.)

The inhibitor was finally isolated by merely scraping the epidermal side of the skin sufficiently to remove all the hair (including any stubble). After such treatment, all skins, regardless of the genotype from which they were taken, produced a reaction slightly under control (auto-oxidized) level (table 4).

TABLE 4

Effect of removing the epidermis.

SOURCE OF EXTRACT	NUMBER OF ANIMALS		$\bar{A}_R$	$\bar{A}_E$
	R	*E*		
Phenotypic whites	12	21	0.82±0.14	0.21±0.04
E−C−FF (full enzyme production)	17	62	0.96±0.06	0.94±0.05
All colored animals showing negligible enzyme activity	20	63	0.90±0.07	0.40±0.04

$\bar{A}$ = mean activity index.
R = epidermis removed.
E = epidermis intact.

Microscopic examination of the scraped and unscraped skins (Plates 1 and 2) revealed that the scraping removed the epidermis, producing an almost perfect cleavage between it and the underlying dermal portions. In some cases a little basal layer was left in certain areas, but otherwise removal was complete and never included much, if any, of the dermis. (Removal of the epidermis, of course, included the presumptive enzyme producing cells of the basal layer.) The inhibitor, therefore, is sharply localized and restricted to the epidermis, while enzyme formation is restricted to the pigment cells themselves.

Attempts to concentrate the active agent

Many methods of extraction were attempted during the course of this investigation. Fat soluble extracts were entirely negative. Dehydration of the skins with acetone was attempted, and a powder was prepared by grinding in a Wiley mill. The extraction of this powder was not successful. A similar powder was prepared after vacuum dehydration. It was also inactive. Finally the different extracts were tested against tyrosine, and the results (including those obtained with the final extract used in the dopa work) were again completely negative, though the attempts were fairly extensive.

Saline and buffer extracts show higher activity than the extract actually

used, but give poor results because they are extremely murky and discolored to begin with, hence difficult to assay colorimetrically.

Thus far, though the presence of an enzyme has often been alluded to, it has not yet been shown to exist except in so far as activity indices above 1.0 (found especially in the delayed tests) indicate a positive agent. The "immediate" results, at least, could be explained by an anti-inhibitor rather than an enzyme system in the traditional sense.

Several attempts were made to concentrate the active substance in order to determine whether or not it actually is an enzyme. These attempts gave positive results. In essence, salting out the globulin fraction of the extract concentrates the active substance. Further purification (by redissolving and salting out several times) and concentration (by adsorption and elution) will increase its activity still more as measured by eye or colorimetrically. Boiling inactivates even the crude extract, as does denaturation of the skins by acetone. At no stage is there any activity towards tyrosine, though polyphenols will react with the enzyme according to reports based on previous extract and frozen-section work using other substrates and interpreted in the light of the present evidence.

This suggests that the enzyme is a polyphenol oxidase such as the one described and prepared by KUBOWITZ (1937), and by KEILIN and MANN (1938). These workers salted out both albumin and globulin and used this combined fraction as the basis for further purification. In all cases of fractional salting out of guinea-pig extracts, albumin alone neither added to nor detracted from the reaction.

SUMMARY

The enzyme.—It is possible to extract a substance from the skins of certain genotypes of the guinea pig that behaves like a polyphenol oxidase in certain respects. This substance has been demonstrated in the globulin fraction and can be concentrated from the crude extracts. Boiling destroys its activity. It is concluded that the substance is an enzyme.

The inhibitor.—A substance which completely inhibits the auto-oxidation of dopa over considerable periods can also be extracted from guinea-pig skins. It is restricted to the epidermis and is normally present there, since it cannot be produced by treating dermis in a similar manner. The inhibitor is not found in the albumin fraction of the extract (as has been reported for the rabbit). There is no evidence that it is affected by any of the known color genes.

Enzyme reaction as related to phenotype.—The enzyme reported here is probably concerned only in the formation of yellow pigment. Its concentration is diminished by those genes normally reducing the intensity of yellow pigmentation and is not at all affected by those reducing only the intensity of brown, sepia, or black. It is present in the skins of brown, sepia, and black guinea pigs at concentrations agreeing with the intensity of pigmentation in the corresponding yellows (*E*— replaced by *ee*) and is, therefore, not the critical factor in determining the kind of pigmentation actually produced when *E*— is present. Only quantitative variations in yellow pigmentation are dependent upon this enzyme.

Enzyme reaction as related to genotype.—Replacements at the *P*, *B*, and *A* loci are not paralleled by any significant changes in the dopa reaction. Likewise, replacement of *E*— by $e^p e^p$ has little effect in black or brown areas of tortoise shell animals. Replacement of *FF* by *ff* (which normally reduces yellow) drastically reduces the concentration of the enzyme. *Ff* is intermediate in this respect (in agreement with the imperfect dominance of *F* with respect to grade of yellow). There is good evidence that *ee* also reduces the concentration. Replacement of *C* by c^k or c^d is compatible with only a low order of activity (paralleling the diluting effect of these genes on yellow coat color), while no activity whatever is possible with extracts from animals in which *C* is replaced by c^r or c^a (that is, in which yellow pigmentation is eliminated) or with those prepared from the white areas of spotted (*ss*) animals.

Comparison of extract and frozen section results.—The genotypic distribution of the enzyme agrees with that of the active principle demonstrated in the pigment cells of the hair follicles by W. L. Russell by means of means of the dopa reaction in frozen section. It does not agree with that of the active principle in the pigment cells of the basal layer of the epidermis as demonstrated by Russell.

Spectra of the melanins.—Light absorption curves plotted through the visible spectrum indicate that there is a difference between black and yellow natural pigment. Black pigment gives a curve identical with that obtained for artificial tyrosine melanin, artificial dopa melanin, and natural pigment obtained from potato skins. The pigment from intense yellow (red) guinea pigs shows definitely less absorption than that from black at the red end of the spectrum.

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